Changes in gut bacterial communities in canaries infected by Macrorhabdus ornithogaster

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Patrizia Robino[1]*, Ilario Ferrocino[2], Giacomo Rossi[3], Andrea Dogliero[1], Valentina Alessandria[2], Lisa Grosso[1], Livio Galosi[3], ClaraTramuta[1], Luca Cocolin[2] & Patrizia Nebbia[1]

[1]Department of Veterinary Sciences, University of Torino, Largo Braccini 2, 10095 Grugliasco (Torino), Italy, [2]Department of Agriculture, Forestry and Food Science, University of Torino, Largo Braccini 2, 10095 Grugliasco (Torino), Italy; [3]School of Biosciences and Veterinary Medicine University of Camerino, Via Circonvallazione 93/95, 62024 Matelica (Macerata), Italy.

*Corresponding author: Tel: +39 011 6709190. Fax: +39 011 6709196.
E-mail: patrizia.robino@unito.it

Abstract

Macrorhabdus ornithogaster is an opportunistic yeast that colonizes the gastric mucosa of many avian species. Until now, no studies have been focused on about the influence of a gastric infection on the balance of the intestinal microbiota of birds. In this study, 44 fecal samples from individual canaries, with and without *M. ornithogaster* infection, were analyzed. The detection of the yeast was evaluated by 18S rRNA PCR.

In order to evaluate the impact of the *Macrorhabdus* infection on the bacterial communities, the culture-independent methods by the use of amplicon based sequencing as well as 16S rRNA-DGGE were adopted.

The different health status of animals affected the relative abundance of the main OTUs, with a greater diversification of the gut microbiota in healthy animals if compared to the infected. In particular, *Lactococcus, Pseudomonas, Acinetobacter, Lachnospiraceae, Propionibacterium* and *Weissella* were found to be characteristic of uninfected animals (FDR <0.05), while *Lactobacillus* and *Candidatus* Arthromitus were characteristic of infected animals (FDR <0.05). Both these taxa have been reported as immunostimulatory involved in immunological disorders.

In infected animals the inferred metagenome assessed by PICRUST clearly showed a positive correlation between the presence of *M. ornithogaster* and KEGG genes related to ether lipids metabolism, already reported to be immunostimulatory by activation of macrophages and to play a pathophysiological role in several immunological disorders.

Finally, our results show an interaction between infection of the digestive tract and intestinal microbiota of pet birds and provide insight about the changing of the complex enteric bacterial community.

Highlights

1 *Macrorabdus ornithogaster* is a gastric yeast that colonizes a wide range of birds.

2 Differences were found between infected and healthy animals in gut microbiota.

3 *Candidatus* Arthromitus was closely associated with infected birds.

4 *M. ornithogaster* can affect intestinal microbiota composition of canaries.

Keywords: M*acrorhabdus ornithogaster,* gastric yeast, gut microbiota, *Serinus canaria,* 16S rRNA-DGGE, 16S rRNA amplicon based sequencing.

Introduction

Macrorhabdus ornithogaster is a frequent opportunistic pathogen yeast with a worldwide distribution and it causes a disease that usually takes a chronic form, called Megabacteriosis or Macrorhabdosis, characterized by high morbidity and low mortality in both wild and captive birds (Gerlach, 2001; Hannafusa *et al*., 2007; Phalen, 2014). *M. ornithogaster* is an anamorphic ascomycetous yeast, class *Saccharomycetes*, that belongs to its own genus (Tomaszewski *et al.*, 2003). It colonizes mainly the gastric mucosa, in particular the mucosal surface between proventriculus and ventriculus of passeriformes, psittaciformes, poultry and other species, causing symptoms especially in immunocompromised birds. Usual clinical signs are weight loss in spite of a good appetite, regurgitation, diarrhea, debilitation, maldigestion, ruffled plumage with stages of recovery and relapse and in severe cases death. Transmission occurs via the oral-fecal route and asymptomatic carriers are presumably the main route of infection. *M. ornithogaster* is difficult to isolate so cultural tests are not routinely used. Usually, the diagnosis is based on observation of symptoms and/or microscopic detection of the organism in feces, crop swabs or proventricular washing (Phalen, 2014, Borrelli *et al*., 2015), and the clinical suspects of disease can be confirmed by molecular method (Tomaszewski *et al.*, 2003).

To the best of our knowledge, no studies have been produced about the influence of a gastric infection on the balance of the intestinal microbiota of birds. Recently, Kienesberger *et al.* (2016) indicated that a gastric infection in animals influences the microbiota and host immune response not only locally in the stomach, but distantly as well, affecting important target organs. Thanks to advances in culture-independent molecular analysis, recent studies in healthy birds described the composition of intestinal microbial community. In particular enteric microbiota has been well characterized in domestic poultry, mostly chickens and turkeys, in order to improve the health and animal productivity (Mancabelli *et al.*, 2016; Boschiero *et al.*, 2018). The gut inhabitants of other bird species, as wild and pet birds, has been less investigated (Xenoulis *et al.*, 2010; Garcia-Mazcorro *et al.*, 2017).

The aim of this study was to investigate the impact of *M. ornithogaster* on the intestinal microbial community of canaries by using PCR-DGGE and next generation sequencing (NGS) technologies.

Materials and methods

Sampling. This study was conducted in a breed collection of canaries (*Serinus canaria domestica)* located in the North West of Italy, between October and December 2016, following the microscopic detection of the typical forms of *M. ornithogaster* on the canaries stool. Briefly, fresh fecal samples were collected by sterile cotton-tipped swab from animals separated in individual cages, smeared on a glass slide and analyzed after Gram staining. Megabacteria appeared as gram-positive rods between 20 to 90 µm long and 1 to 5 µm wide, with rounded ends (Gerlach, 2001). The facility contained 200 birds reared in 24 flight cages and 100 breeding cages. All birds were fed with the same commercial diet, available *ad libitum*, consisting in a mixture of seeds, vitamins and minerals. Also clean water was provided *ad libitum*. None of the animals had been treated with antibiotics during the last 12

weeks prior of the study. The concurrent search for other fecal parasites (particularly coccidia) was negative.

We selected 44 animals between six months and two years of age (mean 10 months and median 8 months), of which 13 symptomatic birds that showed signs of possible megabacteriosis (debilitation, ruffled plumage and diarrhea) while 31 were asymptomatic. Since elimination of *M. ornithogaster* is intermittent, each canary was separated in a different cage, and one fecal deposition per animal was collected over a time span of three weeks, once per week. This period is compatible with a steady state of this chronic disease. All samples were transported to laboratory and stored at -20 °C to be subjected to molecular analysis**.**

DNA extraction. Genomic DNA was extracted from 132 fecal samples (44 birds per three sampling time) using a commercial kit (QIAamp DNA Stool Mini Kit, Qiagen, Hilden, Germany) according to the manufacturer's instruction. DNA concentration and purity were determined with a NanoDrop instrument (Celbio, Milan, Italy) and DNA samples were standardized at 50 ng/µl and stored frozen (−20°C) until use. The three aliquots of DNA collected during the experiment from each birds were pooled together prior to amplification in order to reduce the inter-sample variation.

18S rRNA amplicon fragment. Pooled fecal specimens were tested for the presence of *M. ornithogaster* by 18S rRNA PCR by using primers and conditions described by Tomaszewski *et al.* (2003). Each experiment included a positive control consisting of *M. ornithogaster* DNA, extracted from gastric specimens of dead birds with megabacteriosis.

16S rRNA-DGGE. The fecal microbiota was investigated, through PCR-DGGE analysis targeting the V3 region of the 16S rRNA genes of bacteria as elsewhere described (Ferrocino *et al.*, 2015). A database of DGGE fingerprints was created by using the software Bionumerics version 4.6 (Applied Maths, Sint Marten Latem, Belgium). The similarity distance matrix obtained was used to build partial least-squares discriminant analysis (PLS-

DA) by using the R package mix-Omics (www.r-project.org).

16S rRNA amplicon target sequencing. DNA extracts from fecal samples were used to assess the microbiota by the amplification of the V3-V4 region of the 16S rRNA gene using the universal primers and condition described by Klindworth *et al.*, (2013). Due to the poor DNA quality, among 44 samples, eight were excluded from this analysis.

The PCR products were purified by Agencourt AMPure kit (Beckman Coulter, Milan, Italy), and the resulting products were tagged by using the Nextera XT Index Kit (Illumina Inc, San Diego, CA), according to the manufacturer's instructions. Sequencing was performed with a MiSeq Illumina instrument (Illumina) with V3 chemistry and generated 250 bp paired-end reads according to the manufacturer's instructions.

Bioinformatics and statistical analysis. A 2x2 factorial design with two interventions, the presence/absence of symptoms *versus* infected (PCR positive) and uninfected (PCR negative) animals, was used.

Paired-end reads were first assembled with FLASH software (Magoc and Salzberg, 2011) with default parameters. Joint reads were further quality filtered (at Phred < Q20) using QIIME 1.9.0 (Caporaso *et al.*, 2010) and the pipeline recently described (Ferrocino *et al.*, 2017). Briefly OTUs were cluster at 97% of similarity by the UCLUST clustering methods (Edgar, 2010), and centroids sequences were mapped against the Greengenes 16S rRNA gene database, version 2013. Alpha diversity indices were calculated using the diversity function of the vegan package (Dixon, 2003) and analyzed using the Kruskal-Wallis test to assess differences between groups. Adonis and Anosim statistical tests in R environment were performed on Weighted UniFrac distance matrices and OTU table. A filtered OTU table was generated at 0.02% abundance in at least 10 samples through QIIME. OTU table displays the highest taxonomy resolution that was reached by the 16S data, when the taxonomy

assignment was not able to reach the genus level the family was display. The OTU table was used to build a principal component analysis (PCA) as a function of the infected (*Is* and *Ia* subgroups) and uninfected (*U*) status by using the *made4* package of R. Pairwise Kruskal-Wallis tests were used to find significant differences in microbial taxa abundance according to the groups. P-values were adjusted for multiple testing and a false discovery rate (FDR) <0.05 considered as significant. PICRUSt (Langille *et al.*, 2013), was used to predict the abundance of the inferred KEGG genes as recently described (Ferrocino *et al.*, 2016). Nearest Sequenced Taxon Indexes (NSTI) were calculated in order to evaluate the accuracy of the metagenome predictions (Langille *et al.*, 2013). KEGG orthologs were then collapsed at level 3 of the KEGG pathways and the table was imported into R. Pairwise Kruskal-Wallis tests were used to find significant differences in KEGG pathways according to the groups. Spearman's correlations between OTUs occurring at 5% in at least two samples and inferred metabolic pathways related to amino acid, lipid, energy, and carbohydrate metabolism were taken into account and used to produce a heat plot. Statistics and plotting were carried out in the R environment (www.r-project.org).

Nucleotide sequence accession number. Sequencing data were deposited in the Sequence Read Archive of the National Center for Biotechnology Information (SRA accession number SRP154815).

Results

Samples enrolled in the study. The presence of *M. ornithogaster* was assessed by specific PCR based on 18S rRNA. On the base of the 2x2 study design, the 44 canaries enrolled were divided as follow: 15 uninfected birds (named group *U*, PCR negative samples) and 29 infected birds (group *I*, PCR positive). The group *I* included 16 asymptomatic animals

(subgroup *Ia*) and 13 with signs close related to megabacteriosis: debilitation, ruffled plumage and diarrhea (subgroup *Is*).

DGGE fingerprinting of bacteria community. The DGGE fingerprinting of the V3 region of the bacteria 16S rRNA gene was analyzed through partial least-squares discriminant analysis (PLS-DA) among the groups (Figure 1) and clearly showed the separation among the groups. The distinction was particularly important for samples of group *U*, which appeared to group together and separated from samples of subgroups *Ia* and *Is*.

16S rRNA amplicon based sequencing data. In order to assessed the effect of the presence of *M. ornithogaster* on the gut bacteria microbiota the 16S rRNA based sequencing was performed. The number of libraries obtained for NGS analysis were 36 divided into three groups of animals: unchanged group *U* (15 uninfected animals) and group *I* (21 *M. ornithogaster*-infected animals), split in 9 asymptomatic (subgroup *Ia*) and 12 symptomatic (subgroup *Is*) birds.

A total of 1.932.908 raw reads (2x250bp) were obtained after sequencing. After assembling and quality filtering, a total of 1.829.725 reads passed the filters applied through QIIME, with an average value of 46.916 reads/sample, and a sequence length of 449 bp. The rarefaction analysis and the Good's coverage express as percentage indicated that there was a satisfactory coverage for all the samples (Good's coverage average 89.73%) (data not shown). Moreover, alpha-diversity also showed that there was a higher level of complexity (P <0.05) in *U* samples when compared to *I*. Adonis and Anosim statistical tests based on Weighted UniFrac distance matrix and on OTU table showed significant differences among groups (P <0.001). Differences between groups were further demonstrated by principal component analysis (PCA) based on the relative abundance of the main OTUs (Figure 2). The PCA clearly showed that *U* samples were well separated from *Ia* and *Is* samples. ANOSIM statistical test confirmed this difference (P < 0.01). The effect of age as a covariant influence on the

microbiome was tested with Adonis test with 999 permutation and the results showed no significant difference between samples as a function of the age (P = 0.631).

Microbiota signature in canaries. At phylum level (OTU table filtered at 0.2% in at least 10 samples) we observed that *I* samples were characterized by the predominance of *Firmicutes* (median value 77% of the relative abundance) and *Proteobacteria* (median value 13%) (Figure 3). On the other hand the *U* samples were characterized by the predominance of *Cyanobacteria* (median value 41%), *Proteobacteria* (median value 27%) and *Firmicutes* (median value 19%). When comparing the microbiota at phylum level between *Ia* versus *Is,* we observed that only the minor fraction of the OTUs (< 4% of the relative abundance) identified at phylum level were different between the two samples. *Ia* display the higher abundance of *Acidobacteria* (median value 0.01%), *Actinobacteria* (median value 4.1%), *Cyanobacteria* (median value 2.3%), and *Planctomycetes* (median value 0.01%) (data not shown).

Taking into the account the main OTUs at genus or family level shared between *U* and *I* samples, it was possible to identified a varied microbiota (Table 1) composed mainly by *Lactobacillus* (32±18% of the relative abundance in *I* compared to 6±14% in *U* samples), *Candidatus* Arthromitus (14±4% of the relative abundance in *I* compared to 3±2% in *U* samples), *Enterobacteriaceae* (4±13% in *I* and 2±5% in *U* samples), *Streptococcus* (4±1% in *I* and 0.5±3% in *U* samples), *Clostridium* (0.4±1% in *I* and 6±0.5% in *U* samples). But also a varied minor microbiota was observed.

By comparing the relative abundance of the OTUs across *U* and *I* samples we observed that *Lactobacillus* and *Candidatus* Arthromitus were clearly associated with *I* samples (FDR <0.05) while the minor fraction OTUs was characteristic of *U* samples composed by *Lactococcus, Pseudomonas, Acinetobacter, Lachnospiraceae, Propionibacterium* and *Weissella* (FDR <0.05).

When comparing the microbiota of *Ia* versus *Is* (Table 1), we observed a different microbiota composition. *Lactobacillus*, *Acinetobacter*, *Streptococcus* and *Propionibacterium* were associated with subgroup *Ia* while *Candidatus* Arthromitus and *Clostridium* were associated with subgroup *Is* (FDR <0.05).

Regarding the predicted metagenomes, the weighted nearest sequenced-taxon index (NSTI) for the samples was 0.073 ± 0.010 and indicates a satisfactory accuracy for all of the samples (93%). KEGG genes involved in short chain fatty acid metabolism (propanoate and butanoate), fructose metabolism and D-alanine metabolism were the abundant in *U* samples while KEGG genes involved in glicerophospholipid metabolism were characteristic of the *I* samples (FDR <0.05). When plotting the correlation between OTUs and predicted pathways (Figure 4) it appeared that *Clostridiaceae* and *Lachnospiraceae* were mainly related (FDR <0.05) with inferred genes associated with amino acid metabolism (arginine, proline, phenylalanine and lysine metabolism). *Enterobacteriaceae* were found related with fatty acid metabolism, histidine and starch and sucrose metabolism; *Candidatus* Arthromitus with ether lipid metabolism (FDR <0.05).

Discussion

This observational work presents for the first-time the application of culture-independent NGS analysis to study the fecal microbiome of birds with a gastric infection. *M. ornithogaster* is a gastric yeast that infects a large number of bird belonging mostly to Passeriformes, Psittaciformes, Ratites genera (Phalen, 2014). These animals can be asymptomatically colonized, but the number of reports of outbreaks with high morbidity and mortality is increasing (Razmyar *et al.*, 2016). As in many infections, disease outcomes depend on host genotype, environmental factors (e.g. crowding and hygiene), variation of strains and quantitative load of *M. ornithogaster* (Filippich & Hendrikz, 1998). Recently we have been

able to discover that *M. ornithogaster* is recognized by humoral and cell mediating host immune responses (Rossi *et al.*, 2018a), which could have further effects on colonizing microbiota and host physiology. This infection could predispose to other diseases, and there is substantial clinical and epidemiologic evidence for a negative role in gastrointestinal (GI) disorders outcome (Phalen, 2014).

Our results demonstrate clearly that the group of *U* animals showed a greater diversification of the gut biota compared to the *I* group. *Firmicutes* were found to be the most abundant phylum in *I* group of pet birds, while *Cyanobacteria* and *Proteobacteria* phyla resulted more frequent than *Firmicutes* in *U* animals. These data are not entirely consistent with the few studies on the intestinal microbiome of pet and wild birds (Hird *et al*., 2015; Alcaraz *et al*., 2016; Garcia-Mazcorro *et al*., 2017). These works showed that *Firmicutes* are the most abundant phylum found. Presumably this is due to various causes, as each kind of bird has a very large morphological and ecological differences: relationship among environmental, health, dietary and host taxonomy play an important role in microbiota composition. At this time, the literature regarding the diversity of the microbiota in pet birds is poor (Alcaraz *et al*., 2016; Garcia-Mazcorro *et al*., 2017) and, in particular, it is lacking about changes in microbiota due to infectious diseases.

Among bacteria identified with higher frequency in group *I* we found *Candidatus* Arthromitus, an inhabitant of the intestinal epithelium of mammals, arthropods (Thompson *et al.*, 2012) and birds (Johnson *et al.*, 2018). It is the collective name for segmented, filamentous, non-culturable Gram positive bacteria (SFB). In taxonomy it assumes a distinct lineage within the *Lachnospiraceae*, order *Clostridiales*, phylum *Firmicutes*. SFB are a group that has garnered much attention due to their ability to specifically modulate their host's immune response through the coordination of T cell responses, including the differentiation of T helper (Th17) cells and the induction of IgA plasma cells in the gut lamina propria, and

intestinal IgA-secretions (Umesaki *et al.*, 1999; Ivanov *et al.*, 2009; Goto *et al.*, 2014). In addition, SFB selectively induces the expression of the major histocompatibility complex class II molecules on the intestinal epithelial cells (Umesaki *et al.*, 1999), inducing T-cell-mediated immunological responses. In this respect, strong SFB colonization in mice induces a significant increase in cytotoxic activity of natural killer (NK) cells and CD8+ T cells (Cebra *et al.*, 1998). Given the influence of SFB on many players of the immune response, it seems quite possible that this taxon can interfere with the expression and the activity of some gastrointestinal alterations that contribute to the negative outcome in *M. ornithogaster* infected canaries.

The inferred metagenome clearly showed a positive correlation between the presence of *M. ornithogaster* and inferred metabolic KEGG pathway related to ether lipids metabolism. Ether lipids compounds have already reported to be immunostimulatory by activation of macrophages and to play a pathophysiological role in several immunological disorders (Watschinger & Werner, 2013).

Enterobacteriaceae were also a highly represented group in our infected canaries. It is actually assumed that intestinal dysbiosis is characterized by an overall decrease in microbial diversity with an increase in *Enterobacteriaceae* (Butto *et al.*, 2015).

Faecalibacterium and *Lactobacillaceae* showed trends towards an increase abundance in canaries with *M. ornithogaster* symptomatic infection. The increase of these two taxa in association to SFB positive trend, appeared to be the strongest driver of the microbiota differences between uninfected and infected birds. These taxa are considered as important immune-modulatory bacteria and studies on other animal species (dogs) and humans have shown a higher abundance of *Faecalibacterium* in healthy controls relative to animals with GI diseases (Sokol *et al.*, 2009; Suchodolski *et al.*, 2012). Lactobacilli are often found in the gut microbiome of animals, and they are used as probiotics to promote weight gain in chickens as

well as to protect against some enteric bacteria, such as *Salmonella* or *Campylobacter* spp. (Alcaraz *et al.*, 2016).

In a previous work, we have demonstrated that persistent infection of the proventricular mucosa infected by *M. ornithogaster* initiates an inflammatory cascade that progresses into atrophic gastritis, a condition associated with reduced capacity for secretion of gastric acid and an increased gastric pH in severely affected canaries. This phenomenon is also mediated by an imbalance between gastrin and secretin producing cells in proventricular glands (Rossi, 2000). A reduced capacity for gastric acid secretion allows survival and proliferation of other microbes normally killed by the acidic environment, that are able to pass the gastric tract and colonize the intestinal mucosa. This phenomenon has been very clearly described by Engstrand *et al.* (2013) in human patients severely colonized by *H. pylori*; in these patients a severe atrophic gastritis was associated to a substantial modification of intestinal microbiota with an increase in genera like *Prevotella* or *Streptococcus*. Also Dicksved et al. (2009), during human gastric cancer described an increase in the number of bifidobacteria/lactobacilli, *Veillonella* and streptococci. The respective role of these species in improving or worsening of gastric pathology remains to be determined. A similar situation can also be configured in our case where canaries infected by *M. ornithogaster* showed an increase in enteric colonization by *Streptococcus*, *Fecalibacterium* and *Lactobacillaceae*.

Currently very little is known about the effect of changes in gastric pH on the modification of the composition and properties of intestinal microbiota, and also on the biological and metabolic properties of lactobacilli. However, it has been highlighted as some microbes could be induced to survive even on extreme conditions including acidic environment. Those changes developed by the bacterial strains to survive under adverse conditions are attributed to the phenomenon of "*acclimatization*", which is produced by the expression of different genes (De Angelis & Gobbetti, 2004). In this optic, the selective pressure of acid stress on

lactobacillaceae/probiotic Gram-positive biota not only induces changes on the components of the bacterial membrane, lipids and proteins, but also disturbs the DNA and peptidoglycan components. As result of these phenotypic changes at the cellular level, and the lack of favorable conditions for bacterial proliferation, bacteria presents several mechanisms to avoid stresses, such as, the synthesis of chaperones that act to repair proteins and DNA damage and changes in metabolic pathways to produce alkali and exopolysaccharides (EPS) (De Angelis & Gobbetti, 2004; Lee ~~Yuan~~ & Salminen, 2009; Mayorga-Reyes *et al.*, 2009; Sutherland, 1994). Many *lactobacillaceae* are well known to perform this; these species acquire or increase their "*beneficial*" power only after the gastric pH has induced changes in their metabolic pathways and structure. Naturally, the bacterial EPS confers a barrier of protection to acclimated bacteria against acid pH stress preventing proton diffusion at the intracellular medium (Stack *et al.*, 2010), and contributes to the formation of biofilms allowing colonization in certain substrates providing an ideal microenvironment for unfavorable conditions (Sutherland, 1994; Lee ~~Yuan~~ & Salminen, 2009). Conversely, as observed in our study, we assume that an increase in gastric pH, linked to a strong gastric colonization performed by *Macrorhabdus*, if on the one hand favors an easier survival of intestinal lactobacilli, on the other does not induce their functional EPS-linked modification, greatly reducing its anti-inflammatory, antioxidants, and immunomodulants properties.

This mechanism could explain why in our study, as well as reported in other studies (De Angelis & Gobbetti, 2004; Lee ~~Yuan~~ & Salminen, 2009; Mayorga-Reyes *et al.*, 2009; Stack *et al.*, 2010; Sutherland, 1994; Kanmani *et al.*, 2013), an inflammatory reaction and a change in the secretory pattern of the proventriculum secondary to *Macrorhabdus* infection (Van Herck *et al.*, 1984), produce an apparently paradoxical effect of favoring/promoting enteric colonization by potentially protective *Streptococcus*, *Fecalibacterium* and above all

Lactobacillaceae, but failed in attempt to protect from clinical signs development and worsening clinical conditions of infected birds.

We next used PICRUSt in order to explore the inferred function profiles of microbiota. We observed that uninfected canaries were characterized with putative genes involved in SCFA biosynthesis. The presence of SCFA can be related with many health related functions like anti-inflammatory effects (De Filippis *et al.* 2016) and thus indicated a healthy status of the canaries if compared with the infected subjects that showed lower presence of these genes. Production of SCFAs serves as remarkable source of energy for enterocytes (Sunkara *et al*., 2012) and can suppress gut pathogens (Rehman *et al*., 2007). In addition, SCFAs have been shown to increase parietal tone and stimulate gastrointestinal propulsive contractions when administered into the human terminal ileum (Kamath *et al.*, 1988; Coffin *et al.*, 1997). In this optic SCFAs are the most important metabolites that may accelerate and/or regulate intestinal transit by decreasing toxic intestinal methanogens, since overproduction of methane has been shown to directly inhibit motor activity (Sahakian *et al.*, 2010), and by increasing intestinal fermentation (that enhances intestinal peristalsis and decreases transit time). For instance, an altered intestinal fermentation of a relevant malabsorbed quantity of carbohydrates, as observed in birds with proventricular atrophy and/or dilation (Rossi *et al.*, 2018b), has been shown to significantly increase the number of high amplitude propagated contractions (Jouët *et al.*, 2011), inducing diarrhea or acute intestinal dilation if methanogens prevail. In these conditions SCFAs are good physiological regulators of the manometric intestinal mass movements (Narducci *et al.*, 1987).

Our results show an interaction between infection of the digestive tract and microbiota of pet birds, and provide insight about the changing of the complex enteric bacterial community. Furthermore, these communities cluster separately for each of groups of birds, uninfected and infected. The gastric niche is not isolated, and future longitudinal studies should be carried out

364 to analyze the microbiome of avian species affected by different pathogens, in order to further
365 clarify the pathological importance and the consequences of microorganisms present in the
366 gastro-intestinal tract of birds.
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Figure captions:

Figure 1. Partial least-squares discriminant analysis (PLS-DA) model based on PCR-DGGE similarity matrix. Samples are color coded according to *Macrorhabdus ornithogaster* infected -with or without symptoms- (subgroups *Is* and *Ia*, color coded black and grey respectively) and non infected (group *U*, white) birds.

Figure 2. Principal component analysis (PCA) of the gut microbiota. Samples are labelled according to *Macrorhabdus ornithogaster* infected -with or without symptoms- (subgroups *Is* and *Ia*,) and non infected (group *U*) birds.

Figure 3. Boxplots showing the relative abundance of *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes* and *Proteobacteria* phyla in fecal samples of *Macrorhabdus ornithogaster* infected (*I*, white boxplots) and non infected (*U*, black boxplots) birds. Boxes represent the interquartile range (IQR) between the first and third quartiles, and the line inside represents the median (2nd quartile). Whiskers denote the lowest and the highest values within 1.56 IQR from the first and third quartiles, respectively. Circles represent outliers beyond the whiskers.

Figure 4. Heat plot showing Spearman's correlations between OTUs predicted metabolic pathways. Rows and columns are clustered by Ward linkage hierarchical clustering. The intensity of the colors represents the degree of correlation between the OTUs and KO as measured by the Spearman's correlations with black indicating a positive correlation and white indicating a negative correlation.